Report of Dr. Avery with (Drs. Stillman, Tillett, Julianelle, Goebel, Dubos, Francis, Kelley, and Babers.

Studies on Pneumococcus Infection and Immunity.

I. Further observations on Cutaneous Reactions in Pneumonia with the Specific Polysaccharides and Proteins of Pneumococcus.

II. Development of Heterologous Type-specific Antibodies during Convalescence from Pneumonia.

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XI. Publications.

I. Further Observations on Cutaneous Reactions in Pneumonia with Polysaccharides and Proteins of Pneumococcus.

(Dr. Tillett and Dr. Francis)

In a recent article observations were reported concerning cutaneous reactions obtained in pneumonia following intradermal injections of the type-specific polysaccharides and proteins of Pneumococcus. The results may be summarized as follows: During the acute phase of pneumonia no reaction can be produced with either fraction. Following recovery, however, two responses are obtained of entirely different character. The protein reaction, which reaches its height in twenty-four hours and gradually fades away in a few days, is tuberculin-like. It was elicited in most of the convalescents and its presence or absence appeared to bear no relation to the titre of anti-protein precipitins present in the serum of the patient.

On the other hand, reactions obtained following injections of the type-specific polysaccharides of Types I, II, and III, present several significant characteristics. In the first place, it is a particularly interesting fact that these bacterial sugars, protein-free, are capable of causing definite reactions in humans. In the second place, the character of the reaction is unique in that it appears within a few minutes after injection, is "wheal and erythema" in type and fades away in a few hours. When a positive response was obtained it was always induced by the Polysaccharide homologous in type to that of the infecting organism. For the polysaccharide reaction to occur it has been found that certain conditions

are essential, namely, the patient must have recovered from an infection with the homologous organism and circulating type-specific antibodies must be present.

In continuing this study of cutaneous reactions, these latter points have been given special consideration because of the possible practical applications. One of the practical points is connected with serum therapy in Type I pneumococcus pneumonia. Insofar as our experience has gone, it appears that when a patient, receiving serum, reacts to the intradermal injection of the Type I carbohydrate, it means that recovery has occurred and no more serum is indicated. This principle has been followed in fourteen cases and has been found dependable. Additional evidence of the reliability of the test as an index of recovery is found in the Type I cases which, though treated with serum, died. There have been four instances of this kind. Although sufficient serum was given to maintain an excess of antibodies in the blood of these patients, and although in two cases the temperature was markedly reduced, at no time was a positive cutaneous reaction produced by the Type I carbohydrate. In four cases of Type I pneumonia, in which no serum was given, a positive test was obtained coincident with recovery. From the observations made on individuals ill with Type I pneumococcus pneumonia, it seems justifiable to conclude that skin tests with the Type I polysaccharide are capable of furnishing information of practical significance.

In cases of Type II and Type III pneumococcus infection, the results of skin tests with homologous carbohydrate show that

about fifty per cent of the cases react. The accompanying table summarizes the results. The fact that some individuals recovered from Type II or Type III pneumococcus infection failed to react intradermally, although they possessed circulating type-specific antibodies, indicates that all of the factors involved are not yet understood. Investigation of these conditions is being continued.

	Type I	Type II	Type III	Group IV	Type IIA
No. of cases recovered	18	17	9	13	5
No. of cases recovered giving positive reaction	18	10	4	0	0
Per cent of positive reactions	100	58.8	44.4	0	0
No. of cases not reacting	4	8	7	14	5
Per cent fatal	100	12.5	28.5	7.1	0
Per cent recovered cases not reacting	0	87.5	62.5	92.9	100

II. The Development of Heterologous Type-specific Antibodies During Convalescence from Pneumonia.

(Dr. Tillett and Dr. Francis)

It is well known that in pneumonia, specific antibodies make their appearance at crisis, the time at which the polysaccharide skin test is first demonstrable. In the course of our observations, it has been the custom to test the patients at repeated intervals during the disease and convalescence. Recently it has been noted that about two weeks after recovery a patient may react to a polysaccharide, heterologous in type to the pneumococcus causing the disease. Tests of the serum at the time of such a response have revealed the presence of specific antibodies reactive not only with the primary causative organism but also with pneumococci corresponding type to the "new" specific antibodies. The course of events is

more clearly demonstrated by the accompanying table:

Patient: Roche.

Organism derived from sputum on admission: Type III.

Type-specific Agglutinins	Number of days after crisis												
	Crisis	2	4	6	8	10	12	14	16	18	20	22	24
Type I	-		-		-				-				+++
Type II	-		-		-				+++				+++
Type III	++		+++		+++				+++				++

This patient suffered from Type III pneumococcus pneumonia. At crisis her serum showed only Type III agglutinins. Sixteen days later she possessed demonstrable antibodies against Type II pneumococci as well as Type III. Twenty-four days after recovery there was present in her serum agglutinins and passive protective antibodies for each of the three fixed types.

Since observations of this character were first made, seventeen patients have been followed through convalescence. Of these, ten have developed specific antibodies heterologous in type to that of the infecting organism. The time of appearance of the heterologous reaction has varied from the eighth to the thirty-sixth day of convalescence. The average time has been about two weeks.

Although an explanation of the phenomenon can yet be made, several possibilities suggest themselves. It is interesting to note that the patients who developed heterologous antibodies had received repeated intradermal injections of minute doses of polysaccharides. The possibility that these materials may be antigenic deserves consideration. The recent work of Griffith demonstrating the interconvertibility of type-specific pneumococci also suggests a basis of

explanation. Finally there is the possibility that the distinctive processes of pneumonia resolution may induce slight alterations in some common constituent of the bacterial cells (Fraction "C"?) and that this, acting as antigen, stimulates the development of more than one of the closely allied pneumococcus type-specific antibodies.

III. Serological Reactions in Pneumonia with a Non-Protein Somatic Fraction of Pneumococcus.

(Dr. Tillett and Dr. Francis)

Up to the present time the two chemical constituents of Pneumococcus employed in serological and immunological reactions have been:

1. Soluble Specific Substance (type-specific carbohydrate).
2. Somatic Nucleo-protein (species-specific protein).

The present report is based upon observations made with a third fraction (designated fraction "C") derived from pneumococci, and chemically distinct from the other two. The exact chemical nature of Fraction "C" is being investigated by Dr. Goebel and his results up to the present are reported on pp. of this report. In this communication it is sufficient to state that this substance is non-protein and appears to be a carbohydrate or glucoside common to pneumococci of all types.

Fraction "C" is obtained in the following manner: The organisms contained in a broth culture of degraded, non-type specific pneumococci (R strain) are separated by centrifugation and resuspended in normal salt solution in 60 fold concentration. The bacteria are then frozen and thawed several times until dissolution takes place. 0.3 to 0.5 cc. of normal acetic acid is added and the solu-

tion boiled for 10 minutes. The heavy coagulum thus formed is precipitated by centrifugation and the water clear supernatant fluid is removed and rendered neutral by the addition of the proper amount of normal NaOH. This fluid contains Fraction "C". That Fraction "C" is not a type-specific carbohydrate is indicated by the fact that it is derived from non-type specific R strains of Pneumococcus; that it is not nucleo-protein is indicated by the fact that boiling the material with acid removes protein to such an extent that the supernatant fluid gives none of the usual tests for protein. Although final proof as to its exact nature awaits chemical analysis, nevertheless, convincing evidence of the separate identity of Fraction "C" is brought out by serological reactions.

Sera obtained at frequent intervals from patients acutely ill with or convalescent from pneumonia have been mixed with varying dilutions of Fraction "C" and the presence or absence of precipitation noted. It had been found that serum derived from a patient during the acute stage of pneumonia possesses a high titre of precipitins for Fraction "C". A day or two after recovery this precipitating power abruptly and permanently disappears. The sera of 50 patients have been tested at frequent intervals from admission to the hospital until several months after recovery. In every instance, the blood obtained on admission has furnished serum capable of precipitating Fraction "C" in high titre. This has been true even of patients in the first 24 to 36 hours after the onset of the infection. Individuals who have succumbed to pneumonia have maintained anti-"C" precipitins until exodus. In an individual ill with pneumonia, not only is the sudden appearance of the reactivity striking, but the rapid disappearance of the phenomenon coincident

with recovery is distinctive. A few days after the critical fall in temperature, the patient's serum fails completely to precipitate Fraction "C". The phenomenon may be further characterized by the fact that it is unrelated to the type of Pneumococcus causing infection.

The curve of the precipitin titre of Fraction "C" is distinctly different from that obtained by the use of either the type-specific carbohydrate or the nucleo-protein fractions. With sera obtained at frequent intervals during the course of pneumonia and tested with pneumococcus nucleo-protein, type-specific carbohydrate, and Fraction "C", three distinct curves of precipitin content may be demonstrated. Antiprotein antibodies do not vary markedly during the course of pneumonia. Type-specific antibodies are absent during the acute stage, appear at about the time of crisis, and are homologous to the type of the infecting organism. On the other hand, anti-"C" precipitins are highest during the acute phase of the disease, disappear just after the crisis, and are not related to type-specificity.

The report so far has been limited to a presentation of results obtained with patients suffering from pneumococcus infection. Patients having pneumonia due to hemolytic streptococcus as well as individuals acutely ill with other febrile diseases have been available for comparison. Patients suffering from the following acute diseases have been studied: measles, chicken-pox, acute rheumatic fever, osteomyelitis (staphylococcus) malaria, typhoid fever, tuberculosis, acute gonorrhea, and fevers of unknown origin. Of this group the patients afflicted with hemolytic streptococcus pneumonia, acute rheumatic fever and staphylococcus osteomyelitis, have pos-

sessed anti-"C" precipitins in their serum when bled during periods of acute infection. Tests made with serum from the other cases have given entirely negative results. Through the courtesy of Dr. Swift sera obtained at frequent intervals from fifteen cases of rheumatic fever have been available. Since, in this disease, there are relapses, these cases have furnished instances of inter ittent febrile and afebrile states. By testing sera obtained from such cases it has been found that precipitins for "fraction C" are present during periods of fever but absent during remissions,

These observations made with sera from cases other than pneumococcus infection indicate that the reaction is not specific for pneumococcus pneumonia. It appears, however, to be limited to diseases associated with gram positive cocci.

The significance of the serological reaction which has been described is not yet clear. However, its unusual characteristics both as to time of appearance and rapid disappearance following recovery, indicate that an understanding of the factors concerned in the production of this reaction may throw additional light on some of the problems of acute bacterial infection.

IV. Chemo Immunological Studies on Conjugated Carbohydrate-Proteins.

(Dr. Goebel)

1. Synthetic Carbohydrate (Hexose) Protein Antigens:- In the last report the synthesis of the P-aminophenol glucosides of glucose and galactose was described. It has since been found that when these two carbohydrate derivatives, which differ from one another only in the special configuration of the fourth carbon atom

of the sugar, are bound to protein, these protein-sugar complexes may function as excellent antigens.

It has been found, furthermore, that when these sugar derivatives are bound to the same protein, they exhibit distinct immunological specificity. If, on the other hand, the same carbohydrate radical is conjugated with two chemically different and serologically distinct proteins both of the sugar proteins thus formed acquire a common serological specificity. The newly acquired specificity of these artificially prepared sugar proteins is determined by the chemical constitution of the carbohydrate radical attached to the protein radical. Thus, simple differences in the molecular configuration of a hexose suffices to orient protein specificity when the corresponding glucosides of the sugars are coupled to the same protein. The unconjugated glucosides, though themselves not precipitable in homologous immune serum, specifically inhibit the reaction between the homologous sugar protein and its specific antibody.

Guinea pigs have been passively sensitized with the serum of rabbits immunized with these synthetic sugar proteins. It has been found that they exhibit typical anaphylactic stock when subsequently inoculated with the homologous sugar combined with a protein different from that used in immunizing the rabbit. The reactions are in each instance specific and depend for their specificity on the carbohydrate radical and not on the protein molecule of the synthesized compound.

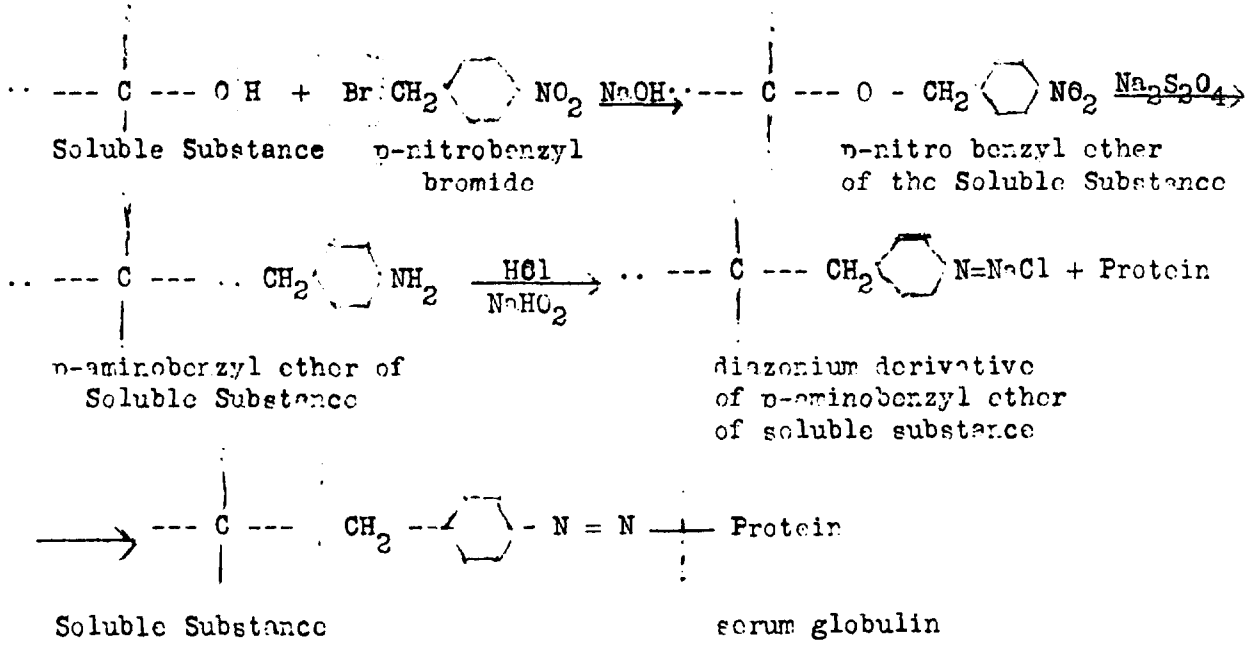
The unconjugated glucosides, though in themselves incapable of inducing shock, inhibit the anaphylactic reaction when in-

jected immediately prior to the introduction of the toxogenic sugar-protein. In order to elicit the phenomenon, the carbohydrate must be the same as that combined in the sugar-protein complex.

Thus, for the first time, it has been shown by direct experimental evidence that asymetry of the carbon atoms in the sugar radical suffices to determine differences in the specificity of sugar-protein antigens.

2. Synthetic Polysaccharide - Protein antigens. For the sake of carrying this conception into the realm of bacterial polysaccharides, where here again it is believed that specificity is dependent upon the arrangement of the atoms and molecules which go to build up the complex polysaccharide, it was thought possible to combine them with foreign protein, and thus to render them antigenic, and to elicit an antibody response similar to that obtained by immunization with the encapsulated bacterial bodies themselves.

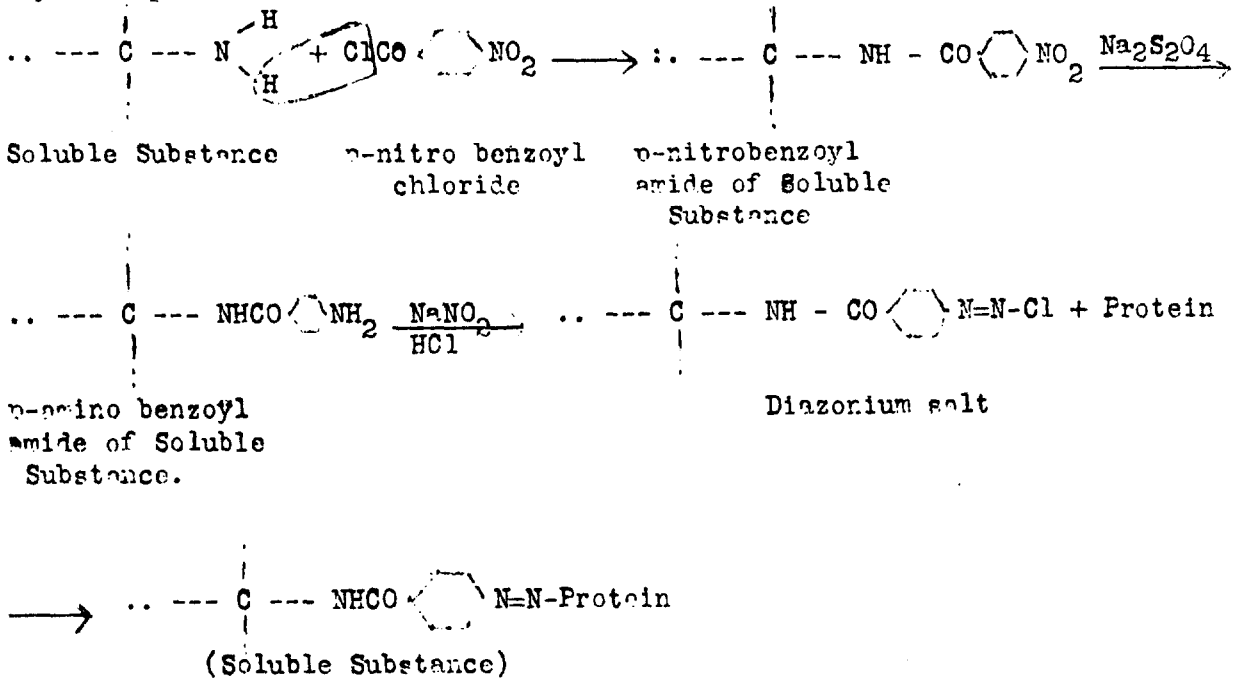
The Type III Pneumococcus soluble specific polysaccharide was chosen for this study. By condensing this compound with p-nitro benzyl bromide an ether of the soluble substance was obtained which, on reduction with sodium hydrosulphite yielded the p-amino benzyl ether. When this compound was diazotized and then added to an alkaline solution of protein (serum globulin), the two substances combined. By chemical manipulation it was possible to separate the unchanged protein from the soluble complex and thus to obtain a conjugated carbohydrate-protein containing neither unbound protein, nor unbound specific polysaccharide. The chemical reactions may be represented schematically thus:



The protein-carbohydrate complex thus obtained was found to be insoluble in the presence of dilute mineral acid, but soluble in dilute alkali. It contained about 10 per cent of bound carbohydrate. It reacted specifically with antipneumococcus serum Type III in dilution of 1:500,000. This conjugated carbohydrate-protein has been used as an immunizing agent and it has been found that successive daily doses of 2 mgs. of antigen given for five days, suffices to elicit a specific antibody response. The sera of rabbits thus immunized, precipitate both the homologous antigen, and the amino benzyl ether of the Type III pneumococcus specific polysaccharide in high dilutions, agglutinate specifically Type III pneumococci, and confer passive protection on mice against infection with virulent pneumococci of the homologous type.

The Type I soluble specific substance of Pneumococcus is an ampholite containing both free amino and free carboxyl groups. It has been found that if an alkaline aqueous solution of this polysaccharide is shaken with a solution of p-nitro benzoyl chloride in benzene, a derivative of this polysaccharide is formed, containing

nitrobenzoyl groups covering the amino groups. This derivative is specifically reactive with Type I antipneumococcus serum in dilutions of 1:5,000,000. When reduced this derivative yields an amine which can be diazotized and coupled to proteins. The chemical reactions may be represented thus:



In a similar manner one may thus attach the Type I soluble substance of Pneumococcus to any protein to yield a conjugated carbohydrate-protein. This derivative will react with Type I antipneumococcus serum in dilutions of 1:500,000. Animals are to be immunized with this derivative and the serological findings will be reported later.

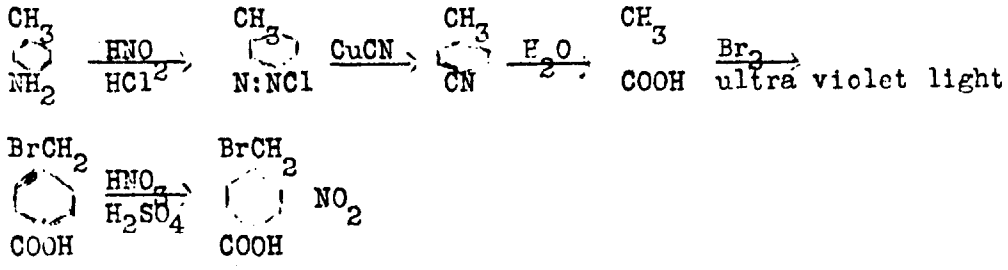
3. The "C" fraction of Pneumococcus: - When cells of an unencapsulated R strain of Pneumococcus are broken up by freezing and thawing, and the resulting solution is heated to 100° C. in the presence of a slight excess of acetic acid, the somatic cellular proteins coagulate. In the filtrates from such coagula there remains a substance, the serological reactivity of which indicates

its separate identity from other fractions of pneumococcus cells. By precipitation with alcohol in the presence of mineral acid, a compound has been isolated which gives none of the usual protein reactions. The material rotates the plane of polarized light about 25° to the right. It is not precipitable by the usual protein reagents. It gives only a very faint biuret test. The substance contains about 5 per cent of nitrogen. It yielded 30 per cent of reducing sugars, calculated as glucose, on hydrolysis. It is not destroyed by proteolytic enzymes. Due to the difficulty in collecting workable quantities, sufficient data has not yet been gathered to characterize this new species-specific substance. It appears, however, to be a polysaccharide. Further investigation is being carried on in order to determine accurately the chemical nature of Fraction "C".

4. Determination of the molecular size of the soluble specific substances: (Dr. Goebel with Dr. Babers). - Experiments are being carried out to determine the molecular size of the specific polysaccharide of Pneumococcus Type III, utilizing as a method the rate of diffusion of the carbohydrate through porous membranes, and determining the minute concentration of diffused polysaccharide colorimetrically. Although this research has not yet been completed, sufficient data have, however, been obtained to indicate that the polysaccharide is of high molecular weight, higher, probably, than most protein.

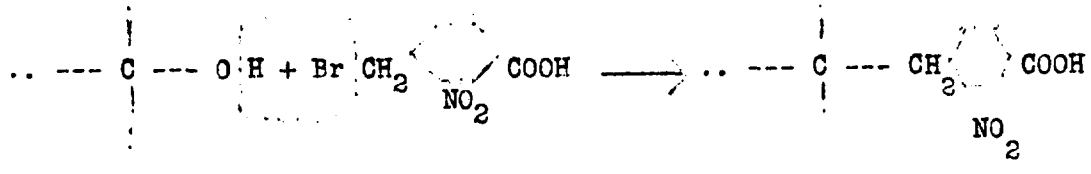
5. Starches as haptens:- The fact that the type-specific polysaccharides of Pneumococcus may be rendered antigenic by combining them with foreign protein has led us to believe that common

starches can also be rendered antigenic by combination with protein carriers. The nitrobenzyl ethers of potato starch and of corn starch have been prepared, but unfortunately these derivatives are totally insoluble in water and in the usual chemical solvents. It was thought, therefore, that the introduction of a carboxyl group into the ring of the condensing reagent nitrobenzyl bromide, would render the corresponding starch ethers soluble in water. Consequently a bromo nitrotoluic acid was synthetized in the following manner from p-toluidine:



Brom nitro toluic acid

This compound has been condensed with starch;

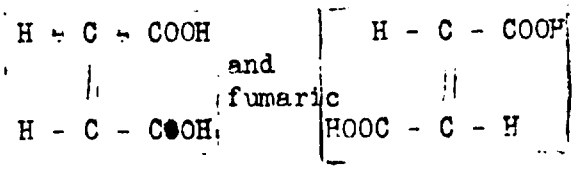


to yield a starch ether readily soluble in dilute alkali, but insoluble in acid. By reducing the nitro group to the amino group and by diazitizing the latter and coupling it with protein, a conjugated starch-protein derivative has been prepared. Immunization of animals with this conjugated carbohydrate-protein derivative are to be carried out to determine whether starches may function as haptens.

6. The relationship between stereo isomerism (geometrical isomerism) and Specificity: - In paragraph 1 of this report it was shown that hexosides which differ from each other only in the spati-

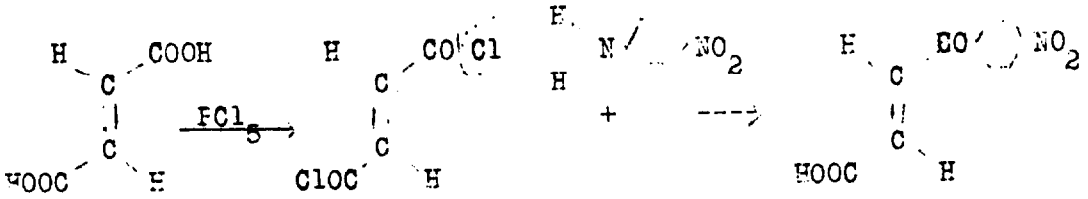
al configuration of one asymetric carbon atom elicit different antibody responses despite the fact that the chemical configuration of the remainder of the hexos molecule is in each instance identical. In order to ascertain whether this phenomenon is confined to compounds containing only asymetric carbon atoms it was decided to study the antigenic response elicited by isomers of the cis-trans type.

Maleric and fumaric acids are the classical



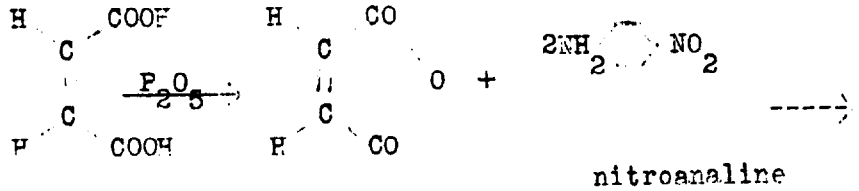
example of this type of isomerism, and they were chosen to serve as the haptens. In order to couple these acids to protein it became necessary to synthesize the mono nitro analides of these acids. This has been accomplished by the following synthesis:

(1)

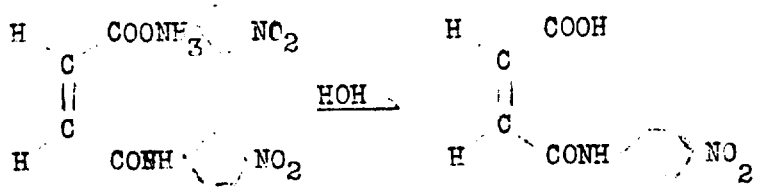


Fumaric acid fumaryl chloride + nitro analine

(2)

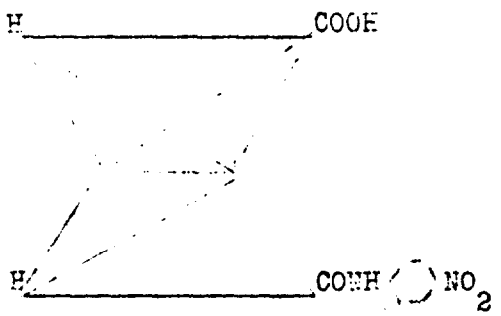


Maleic acid Maleic anhydride nitroanaline

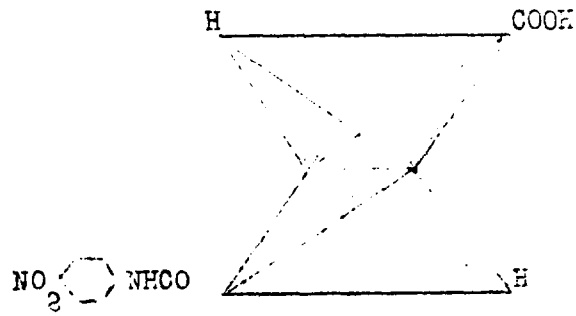


p-nitroanaline salt of p-nitromaleanilic acid p-nitro maleanilic acid

These two compounds differ one from the other not in structure but in the geometrical relationship of their atoms. The isomerism can best be understood from the following diagrams:



p-nitro maleanilic acid



p-nitro fumaranilic acid

The nitro group of these two isomers, which contain no asymetric carbon atoms, have been reduced and the corresponding amines have been coupled to proteins. Animals are to be immunized with these derivatives to ascertain whether geometrical isomerism can effect specific antibody response.

V. Reactions of Rabbits to Injections of Pneumococci and their Products.

(Dr. Julianelle).

A study has been made of the changes which take place in rabbits following the repeated intracutaneous injection of suspensions of heat-killed pneumococci. For the sake of comparison, a similar study has been made in normal rabbits and in rabbits treated with pneumococci and their products in various ways. The reactions investigated have been (1) the antibody response, (2) resistance to infection, (3) the reactions at the site of injection, (4) the development of skin reactivity to derivatives of Pneumococcus, (5) the development of eye reactivity, and (6) hypersensitiveness to Pneumococcus and its products. Each of these reactions is summarized in the present report.

I. The Antibody response. Sixty rabbits were immunized by the repeated injections into the skin of small doses of heat-killed pneu-

mococci, Type I. In the sera of 53 of the rabbits no type-specific antibodies were demonstrated, while in the sera of the remaining animals, the titre of type-specific antibodies was very low. In all cases, however, the sera possessed high titres of the species-specific antibodies.

Forty-five rabbits similarly immunized by injections of heat-killed Type III pneumococci also failed to form type-specific antibodies, but did form species-specific antibodies. Moreover, heat-killed suspensions of R pneumococci or solutions of the bacterial substances when injected into the skin stimulated the production of only species-specific antibodies.

II. Resistance to infection. Following the intracutaneous injections of heat-killed S or R pneumococci, rabbits acquire a marked degree of resistance to intravenous injections of virulent organisms, and this is true whether the pneumococci injected be of the same type or of a different type from that employed for immunization. On the other hand, repeated intracutaneous injection of the soluble proteins of the cell is not followed by an increased resistance to infection. The sera of both resistant and non-resistant animals, in general, fail to protect white mice against infection by organisms of the homologous type. However, the sera of about 20 per cent of the rabbits injected intracutaneously with Type I Pneumococcus, were found to contain varying quantities of protective antibodies. It is seen, therefore, that while the antibody response to the intact cells and solutions of the cell is essentially the same (i.e. species-specific) the acquisition of resistance to infection is characteristic of only the animals receiving the intact cells.

III. The reaction at the Site of Injection. The intracutaneous injection in normal rabbits of 0.2 cc. of heated pneumococci, representing the bacteria from 2 cc. of broth culture is followed by a circumscribed, slightly raised, and indurated nodule, measuring about 1 cm. in diameter. Upon repeated injection of the same amount of bacterial suspension at weekly intervals, the reaction becomes more intense in character and greater in size until 4 to 6 injections have been made, after which the reactions become increasingly milder. In the more intense reactions, the size increases to 4-6 cm. in diameter, and the skin is markedly elevated and of a deep red to purplish hue. The raised area is surrounded by an areola of erythema and outside of this the skin may be edematous over a considerable area. Frequently necrosis of the skin occurs with discharge of a sterile, purulent material. When necrosis does not occur, the disappearance of the lesions is delayed and the time required for regression is related to the intensity of the reaction. This heightened skin reactivity to the bacteria is probably dependent upon some alteration of the tissues themselves since transfers of serum from highly reactive to normal rabbits does not endow the latter with the property of increased activity.

It should be pointed out that frequently a secondary reaction may occur following the disappearance of the primary reaction to the first injection. This recrudescence occurs even without a second injection and is probably evidence of the development of hypersensitiveness.

IV. The development of skin reactivity to derivatives of Pneumococcus. Following a series of intracutaneous injections of heat-killed pneumococci, rabbits acquire an increased skin reactivity

(1) to the nucleo rotein of Pneu ococcus and (2) to an extract of the bacteria from which the acid-preci itable and heat coagulable proteins have been removed. In terms of bacterial specificity this skin reactivity must be considered as species-specific, and it appears to be related to the presence of circulating species-specific antibodies.

A similar skin reactivity has been proved to occur in rabbits following the repeated administration by the intravenous or intracutaneous route of the heat-killed bacteria or their protein derivatives. The skin reactivity, therefore, occurs in both resistant and non-resistant animals.

V. The development of eye reactivity to derivatives of Pneumococcus. It was also found that certain of the rabbits become eye reactive after receiving intracutaneous injections of intact cells. If the cornea is scarified and then the nucleoprotein of Pneumococcus is placed in the conjunctival sac, in about 60 per cent of the rabbits an eye reaction appears within 24 hours and then increases in intensity for varying periods. The reaction consists of congestion of the conjunctiva and the appearance of dilated capillaries at the sclero-corneal margin. In some rabbits, the cornea is also involved and there is the development of turbidity and, less frequently, the formation of a pannus. The eye reaction also is species-specific. It has been found that not infrequently, the intravenous injection of nucleoprotein, after all evidence of the eye reaction have completely disappeared, may cause the reappearance of the eye reaction. In contradisctinction to the skin reaction to protein, the eye reactions do not occur in rabbits fol-

lowing intravenous i unization with the intact cell, or following im-unizati n by any route with solutions of roteins of the cell. So that while skin reactions occur in non-resistant and resistant ani-als alike, the eye reaction, on the other hand, occurs only in the resistant rabbits.

VI. Hypersensitiveness to Pneu ococcus and its derivatives.

There were certain reasons for believing that the skin reactivity to the bacterial protein resembles the Arthus reaction, while the eye reactivity seems to depend upon some factors which are different from those operative in the skin reactivity. It seemed advisable, therefore, to study simultaneously the Arthus reaction to egg albumin, the development of increased skin reactivity to the bacteria themselves and to the protein of Pneumococcus, and the develo ment of eye reactivity. A summary of this comparative study is presented in the accompanying table. (See Table I). The important points in this table not already mentioned are (1) that the skin reactivity to pneumococcus protein can be transferred by serum from a reactive to a normal rabbit; (2) on the other hand, the eye reactivity cannot be thus transferred. In other words, the skin reactivity appears to be an example of the Arthus reaction to a bacterial protein, while the eye reactivity appears to be a special type of sensitivity.

VI. Active Immunity to Pneumococcus Infection Following Injections of the Soluble Specific Substance.

(Dr. Julianelle).

Caspar and Schiemann originally called attention to the antigenicity of the Soluble Specific Substance of Pneumococcus. In a more recent communication, Schiemann repeated the study of the immun-

Table I.

Reactions of Hypersensitiveness in Rabbits

Reactions	Following repeated injections of egg albumin		Following repeated injections of Pneumococcus protein		Following repeated injections of heat-killed pneumococci (Type I)	
	Intracutaneous	Intravenous	Intracutaneous	Intravenous	Intracutaneous	Intravenous
Reaction at site of each injection	1st 3 inject.- 4th - 10th "+		1st 3 injec.- 4th - 10th"+		1st injed.+ Increase in intensity until 4th-6th injed. Then decrease in reaction	
Development of circulating antibodies (a)type-specific	-	-	-	-	-	+
(b)species specific	+	+	+	+	+	+
Active Resistance			-	-	+	+
Increased sensitivity (a) skin	+	+	+	+	+	+
(b) eye	-	-	-	-	+	-
Transfer of sensitivity (a) skin	+	+	+	+	+	+
(b) eye	-	-	-	-	-	-

+ indicates presence of reaction

- indicates absence of reaction

ity of mice to pneumococcus infection following injections of the carbohydrate derived from Pneumococcus, Type II. He pointed out that the immunity developed only when very small quantities of the polysaccharides are injected and the resulting immunity is type-specific.

Experiments have been undertaken to determine, therefore, whether the polysaccharides of the three types of Pneumococcus are antigenic, in the sense that they will stimulate an active immunity; and if so, what quantity of the polysaccharides is best for this purpose.

It was found that the carbohydrates derived from Type I or Type III, irrespective of the method chosen for injection and of the quantities used, did not stimulate in mice a state of resistance to Pneumococcus infection. The total quantities of carbohydrate studied varied in each case from 5.0 to .00005 mgm.

When Type II polysaccharide was employed, it was found that immunity to infection could be induced in mice when small quantities of the soluble specific substance were injected. The total quantities injected varied from 5.0 to .0001 mgm., but only amounts of from .005 to .00005 mgm. stimulated any degree of immunity. The optimum dosage was .001 mgm. In the last instance, the immunity induced was comparable to that induced by the injection of heat-killed Type II pneumococci. The immunity, moreover, was highly type-specific. Rabbits and guinea pigs were also injected with different quantities of Type II polysaccharide, but it was not possible to show that either of these two species had acquired type-specific immunity.

In conclusion, it maybbe said that with the quantities employed, no immunity was demonstrated following injections in mice of

Type I or Type III carbohydrates. When injections were made with minute quantities of Type II polysaccharide, a definite degree of immunity was demonstrated in mice to infection by organisms of the homologous type. Immunity was not demonstrated in rabbits and guinea pigs, following the injection of Type II soluble specific substance.

VII. Immunity Induced in Rabbits by Inhalation of Virulent Pneumococci.

(Dr. Stillman)

Studies planned to determine the relation of virulence to susceptibility of rabbits to infection and to determine the nature and character of antibody response following inhalation of live pneumococci are still in progress. Rabbits are susceptible to fatal infections following inhalation of Type I pneumococci. Type II pneumococci whose virulence for rabbits has been increased by animal passage, and by certain naturally rabbit virulent strains of Type III pneumococci. Rabbits are not subject to fatal infection following spraying with the usual strains of Type II pneumococcus, or with the majority of strains of Type III pneumococcus. The results of the infections following inhalation of pneumococci vary in direct proportion to the virulence for rabbits of the strain used. Some rabbits will even recover from a transient septicemia due to virulent Type I or Type II pneumococci, but in the case of rabbits exposed to inhalation of virulent Type III pneumococci, if the blood is once invaded, a fatal septicemia always ensues.

Following inhalation of Type I pneumococci, agglutinins and protective antibodies are apt to appear in the rabbit's sera.

After spraying with a strain of Type II pneumococcus which has been rendered rough and avirulent, neither of these antibodies occur. If rabbits are repeatedly sprayed with a slightly virulent pneumococcus Type II S. protective antibodies only appear in the blood. If a rabbit virulent Type II pneumococcus, however, is used for inhalation both agglutinins and protective bodies may develop. Following inhalation of either a non-virulent or a highly rabbit virulent strain of Type III pneumococci, neither agglutinins nor protective antibodies appear in the rabbit's blood.

The sera of 2 rabbits which have survived treatment and are now living, almost 4 years (1310 and 1360) days after their last exposure to Type I pneumococci, still protect mice against infection of large doses of a virulent culture of the same type as that with which they were originally sprayed.

VIII. Antipneumococcus Protective Action of Normal Pig Serum.

(Dr. Kelley)

Bull and McKee have reported experiments in which mice and guinea pigs were protected against many times the lethal dose of pneumococci by the injection of normal chicken serum. It appeared that this protective action of the chicken serum was associated with the serum globulin.

In studying phagocytosis of pneumococci by serum-leucocyte mixtures Robertson and Sia observed a marked opsonic activity of the sera of naturally resistant animals. This was found to be especially characteristic of the serum of the pig. Sia observed that normal pig serum would also confer on mice a remarkable degree of passive protection against pneumococcus infection. In cases of Type I and

II Pneumococcus, protection against 10,000 fatal doses was obtained. He also demonstrated by absorption experiments that this protective action was specific for each type of Pneumococcus. At variance with the widely accepted cellular theory of natural immunity, here are instances of a naturally occurring humoral defense mechanism. The immune properties of the serum are passively transferable and appear to be type-specific in action.

The study in progress is an attempt to repeat the work of Sia and to further analyze the mechanism of the protective action of pig serum. The serum is obtained from the blood of normal pigs collected at slaughter, and is sterilized by Berkfeld filtration.

As found by Sia, the fresh serum given intraperitoneally protects mice regularly against infection with 1,000 to 10,000 fatal doses of Type I and Type II pneumococci. The degree of protection varies somewhat with the individual lots of serum. With the sera so far studied the greatest degree of resistance to infection has been conferred on mice against infection with Pneumococcus Type II. This protective capacity diminishes gradually on standing so that in the lots tested it was completely lost after 6 to 12 weeks. There is a suggestion that the protective property is conserved by storing the serum at a low temperature and by covering it with a vaseline seal. Whether the protective powers of the serum against all the different types of pneumococci disappear simultaneously has not yet been determined. Heating the serum at 60-62°C. destroys all, or very nearly all, of its protective action. Under the same conditions antipneumococcus horse serum shows no appreciable loss of protective power. The addition of 10 per cent untreated pig serum, previously inactivated by heating, fails to restore the protective action.

The optimal protective dose of the pig serum seems to lie between 0.75 cc. and 1 cc. As Sia reported, the greatest degree of protection to mice is afforded when the serum is injected intraperitoneally 4 hours before the infecting dose of Pneumococcus culture is given. The protection, though quite distinct, is not of the same degree when the serum is given simultaneously with the culture.

The observations that the protective factor is associated with the globulin fraction of the pig serum has been confirmed. No appreciable amount of this capacity seems to be lost in the process of separation of the globulin by precipitation with ammonium sulphate.

In our experiments so far, absorption with virulent pneumococci of a given type has removed the property of protecting mice against infection with the homologous type. In addition, the degree of protection afforded by the absorbed serum against heterologous types of pneumococci is decreased. A surprisingly few pneumococci are required for the absorption. The time necessary is likewise short. There is a suggestion that prolonged absorption with a large number of virulent or avirulent pneumococci may completely deprive the serum of its power to protect mice against infection with either homologous or heterologous types of Pneumococcus. Preliminary studies in which Pneumococcus polysaccharide was added to the serum 24 hours before the protection tests were made indicate that the serum has not been altered in its protective power by this treatment.

Agglutination and protection tests have been made with a serum which protects mice against 10,000 lethal doses of Type I or Type II pneumococci. Despite its protective action, the serum failed to cause agglutination of Type I or Type II pneumococcus. The precipitin tests, using the pneumococcus polysaccharides derived from Type I

Type II pneumococci, have likewise been negative.

So far, attempts to degrade a virulent S strain of Pneumococcus to an avirulent R form by growth in pig serum have not been successful. Pneumococci sensitized by the serum show no loss of virulence. It has not been learned whether or not sensitization of pneumococci in pig serum has any effect on their agglutinability in anti-pneumococcus horse serum.

In view of the fact that certain substances, of themselves not antigenic, when injected intravenously into animals along with pig serum will stimulate antibody response, it is thought desirable to learn if such "Shlenne" action may be obtained in the case of pneumococcus polysaccharides. Rabbits are being immunized with mixtures of pig serum and the polysaccharide of Type II pneumococcus to determine whether, under these conditions, the non-antigenic sugars may stimulate the formation of type-specific antibodies.

IX. Significance of Oxidation-reduction Phenomena in the Bacterial Cell.

(Dr. Dubos)

1. The role of peptone and glucose in the initiation of growth of Pneumococci:- Preliminary analyses of the oxidation-reduction system of the Pneumococcus cell, and of the oxidation-reduction characteristics of sterile bacteriological media, have been discussed in a previous report.

On the basis of the data obtained and of certain growth experiments in media treated in various ways, it was suggested that: (a) the growth of Pneumococcus is conditioned by the existence in the medium of a certain condition of reduction, (b) there are present in ordinary media certain products of oxidation which have a bacteriostatic action on Pneumococcus. It was found that this bacteriostatic action can be overcome by various methods; addition of reducing substances, incubation

under anaerobic conditions, addition of blood, heating the broth previous to inoculation and by using a large inoculum.

The work of the last year has confirmed and extended these observations. It appears that, when solutions of peptones are kept under aerobic conditions, they become bacteriostatic for Pneumococcus. This bacteriostatic power does not develop when peptone solutions are kept under vaseline seal. The bacteriostatic power varies with different peptones; it is for instance, 3 to 4 times greater with Fairchild's than with Witte's peptone. The bacteriostatic action of the peptone solutions may be prevented by the addition of small amounts of reduced thio-acids, by heating in the presence of glucose, by the addition of heated glucose, or by incubation in the presence of glucose under anaerobic conditions.

Studies concerning the mechanism of the action of glucose are still in progress, they seem to indicate, that under the conditions of our experiments, the reducing properties of the glucose solution are much increased, as a result of a rearrangement of the glucose molecule or the formation of new substances from it.

These experiments indicate that the beneficial action of heating broth previous to inoculation with anaerobes is due not only to the mechanical removal of the oxygen in solution but also to the action of the glucose on the peptone.

2. The role of carbohydrate in biological oxidations and reductions. Experiments with Pneumococcus. We have been that a large inoculum of Pneumococcus culture can overcome the bacteriostatic action of peptones. This can be accounted for by the actively reducing system which is formed when Pneumococcus cells are placed in the

presence of plain broth. An analysis of this system has given the following results.

The washed cells of Pneumococcus are able to reduce the various indicators of oxidation-reduction potentials in the presence of glucose. Oxidized thiol compounds (glutathione, cystine, oxidized thioglycollic acid) are likewise rapidly reduced by glucose in the presence of washed cells of Pneumococcus.

The Pneumococcus-glucose system is able to form peroxide under aerobic conditions. Those substances which form peroxide in the presence of Pneumococcus cells are also the ones which are active in changing hemoglobin into methemoglobin under the same conditions.

The power of washed cells of Pneumococcus to reduce methylene blue in the presence of glucose is dependent on at least 2 constituents of the cell. One of these can be readily removed from the cell by washing. The other is removed or inactivated much more slowly by the process of washing and is destroyed by heating for 10 minutes at 55° C.

These observations indicate that the expression "reducing power of bacterial culture" must be used cautiously, since this reducing power is dependent not only on the nature of the bacterial species, but also on the presence of definite metabolites.

They also indicate that the cell-glucose system can act as a reducing agent, which can correct the bacteriostatic action of the peptone, thus permitting the growth of Pneumococcus even in an unfavorable medium, provided the inoculum be large.

3. The role of oxidation-reduction processes in bacterial variation. A culture of Type III Pneumococcus, maintained at 39° C. in media containing 5 per cent beef serum, or 5 per cent horse plasma,

has been observed to undergo dissociation, most of the organisms changing to R forms after 2 weeks incubation. The same culture, in the same medium, at the same temperature, but under vaseline seal, remained unchanged (100 per cent S) after the same length of time.

On several occasions, we have been able to revert an R culture derived from Type III Pneumococcus, to a typical, encapsulated. and virulent S culture on repeated transfers in the following medium, plain broth + .3 per cent glucose + .05 per cent thioglycollic acid, provided the culture be kept under vaseline seal.

The conditions required for reversion are not, as yet clearly defined and we are not always successful in repeating these experiments. However, there is no doubt that the state of oxidation-reduction of the medium is one of the factors involved in the reversible change from the capsulated, virulent, smooth Pneumococcus to the R variant.

X. The Decomposition of the Specific Polysaccharide of Type III Pneumococcus by a Bacterial Enzyme.

Previous studies from this department have established that the specific polysaccharides of Pneumococcus are not decomposed by any of the body enzymes, nor are they attacked by the common bacterial, actinomyces and molds. These polysaccharides have in particular been found resistant to all known carbohydrates splitting enzymes. We have succeeded, however, in isolating a microorganism that decomposes the specific polysaccharide of Type III Pneumococcus in a medium containing only mineral salts. This organism is a minute, spore forming, gram negative bacillus, which passes through a Berkefeld V filter. Its action is very specific. It attacks only the specific polysaccharide of Type III Pneumococcus, but not that of Type I and Type II, and

does not ferment ordinary sugars. This microorganism gives rise to an extracellular enzyme which also decomposes the specific polysaccharide. The decomposition of the polysaccharide leaves reducing sugars which no longer react with Type III antiserum.

This loss of reactivity, and the specificity of the enzymatic action on only one of the type polysaccharides, is a further proof that these polysaccharides, and not impurities carried along with the , are really the substances responsible for specificity. This had been shown previously by the disappearance of the reaction of the polysaccharide with specific antisera following chemical hydrolysis. But this treatment was of course a very drastic one and could have affected at the same time the hypothetical impurities. It is not likely that such an objection would be justified in the case of the much milder action of the enzyme.

It will be interesting to determine whether the addition of the hydrolysing enzyme to a medium seeded with encapsulated pneumococci will affect the formation of the capsule (which is known to consist largely of the specific polysaccharide).

We are also considering experiments to determine whether the injection of the specific enzyme into susceptible animals will increase their resistance to infection with Type III Pneumococcus by rendering the cells more vulnerable to phagocytosis.

XII. Publications.

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